

# Detection of *C trachomatis* in urogenital specimens by polymerase chain reaction

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## Abstract

**Objective**—To establish a polymerase chain reaction (PCR) protocol for the detection of urogenital *C trachomatis* infection and to compare it with the detection in cell culture.

**Specimens**—Urethral specimens were collected from 62 male patients and cervical specimens from 106 female patients.

**Setting**—Department of Dermatology and Venereology, Ruprecht-Karls-Universität, Heidelberg.

**Methods**—Urogenital specimens were simply boiled for 15 minutes and subsequently subjected to amplification without prior extraction of nucleic acid. The DNA sequence selected for amplification is located in the third open reading frame of the ubiquitous *C trachomatis* plasmid pCTT1. The amplified products were demonstrated by agarose gel electrophoresis followed by Southern blot hybridization. In addition, specimens were investigated with cell culture.

**Main outcome measures**—Results of PCR and cell culture.

**Results**—PCR detected all *C trachomatis* serovars relevant for urogenital infections (D-L2). Serial dilution experiments revealed that the PCR procedure was 100 fold more sensitive than cell culture. The investigation of 168 urogenital specimens showed that the PCR confirmed all 30 cell culture positive results, however, out of the 138 cell culture negative specimens 16 were positive using the PCR.

**Conclusions**—A substantial number of urogenital *C trachomatis* infections detectable by PCR may be missed by the cell culture technique.

## Introduction

For the detection of *C trachomatis* infections in the urogenital tract a variety of methods have been developed. These include different staining procedures to demonstrate intracellular inclusion bodies,<sup>1-4</sup> direct detection of *C trachomatis* antigens by fluoresceine-labelled monoclonal antibodies<sup>5-7</sup> or by enzyme immunoassay<sup>7-9</sup> as well as direct nucleic acid hybridization procedures.<sup>3,10,11,12</sup> However, detection of *C trachomatis*, still has its limitations. If specimens with only few chlamydial organisms are screened, methods of direct antigen detection and direct hybridization techniques reach their limits. When the viability of the infectious agent has suffered, for example, by poor transportation conditions, cell culture fails to detect *C trachomatis*.<sup>13</sup> Therefore, none of the available techniques have the optimal sensitivity to analyse the actual prevalence of *C trachomatis* infections.

The recently developed PCR technology allows the detection of even smallest amounts of specific nucleic acids using repeated amplification steps of a respective target sequence.<sup>14,15</sup> PCR protocols for the detection of *C trachomatis* have been described.<sup>20,21</sup> However, owing to prior extraction of DNA these are rather laborious and not suitable for routine screening. Furthermore, DNA extraction enhances the risk for contamination. Here we describe a simplified PCR protocol using boiled cells from genital specimens without prior extraction of nucleic acids. As in an earlier study<sup>20</sup> the target sequence was selected from the obligatory *C trachomatis* plasmid pCTT1. The protocol of the present investigation proved to be specific and about 100 fold more sensitive compared with the commonly used cell culture technique. Because of its simplicity and sensitivity it may be useful also for the routine diagnosis of *C trachomatis* infections.

## Materials and methods

**Clinical specimens** The specimens tested by PCR were collected from 62 male patients (urethral specimens) and 106 female patients (cervical specimens) of the STD outpatient clinic at the Department of Dermatology at the University of Heidelberg. Thirty of the specimens were found positive and 138 were found negative by cell culture. For the investigation of specimens in cell culture,

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McCoy cells were cultured on glass cover slips in Roosevelt Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal calf serum. After growth to monolayers, cell cultures were treated with cycloheximide at a final concentration of 1 µg/ml. Cultures were then inoculated with 0.2 ml transport medium, centrifuged at 3000 *g* for one hour, the medium changed after two hours, and then incubated for up to 72 hours. To detect inclusions cells were fixed in methanol for ten minutes and 20 µl of fluoresceine conjugated monoclonal antibody (Syva-Merck, Darmstadt) applied. After being incubated in a moist chamber for 15 minutes at 37°C, cover slips were rinsed with distilled water, air dried, mounted and examined with a Zeiss fluorescence microscope.

**Polymerase chain reaction** 200 µl of transport medium were centrifuged for one hour at 3000 *g* at room temperature. The pellet was resuspended in 7 µl transport medium, boiled for 15 minutes and subsequently subjected to PCR in 1 × Taq polymerase buffer [67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl<sub>2</sub>, 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM β-mercaptoethanol, 6.7 µM EDTA (pH 8.0), 0.17 mg/ml bovine serum albumin]. 7.5 U Taq polymerase (Pharmacia, Freiburg, F.R.G.), nucleotides to a final concentration of 10 mM dATP, dTTP, dCTP and dGTP and 200 ng of the respective primers were added and the reaction mixture was covered with 50 µl paraffin oil before amplification. Primer I consisted of the sequence 5'-AAT AGA AAA TCG CAT GCA AGA TAT-3' corresponding to nucleotide position 1596-1620 and primer II of the sequence 5'-CGA GCC AGC ACT CCA ATT TC-3' corresponding to nucleotide positions 2124-2105. The PCR was performed in a DNA thermal cycler (Bühler, Stuttgart) starting with an incubation of 10 minutes at 90°C (denaturation to single stranded DNA), followed by 40 cycles of 3 × 1 minute at 50°C (annealing of the primers), at 70°C (extension of the DNA) and again 90°C. Forty cycles were run through, ending with the last cycle at a temperature of 70°C for 10 minutes.

**Southern blot investigations** Half of the reaction mixture was subjected to electrophoresis in a 2% agarose gel. The DNA bands were visualised after ethidium bromide staining under UV-light. The agarose gel was then incubated three times in 0.25 M HCl for 15 minutes and subsequently rinsed in distilled water. The DNA in the gel was denatured by incubation for 20 minutes in 0.5 M NaOH, 1.5 M NaCl and blotted on a Genescreen Plus™ blot membrane (NEN Du Pont, Dreieich). Upon transfer of the DNA the membrane was neutralized in 3 M NaCl, 0.5 M Tris-HCl (pH 6.0) for ten minutes and subsequently dried at room temperature.

A diagnostic oligonucleotide consisting of the sequence 5'-TCT ATT CGC AGC GCT AGA GG-3' corresponding to nucleotide positions 2054-2073

and located between the amplification primers was labelled by phosphorylation at the 5' terminus with <sup>32</sup>P dATP (Amersham Buchler, Braunschweig) and T4 polynucleotide kinase according to standard procedures.<sup>16</sup> Southern blots were prehybridised overnight at 55°C in 0.9 M NaCl, 6 mM EDTA, 90 mM Tris HCl (pH 7.5), 1% sodium laurylsulfate (SDS) and 100 µg/ml tRNA (Boehringer, Mannheim). Labelled oligonucleotide (10<sup>7</sup> cpm) was added and hybridised overnight. The membranes were first washed for 10 minutes at 55°C in 3 × SSC, 10 mM sodium hydrogen phosphate (pH 7.0), 5% SDS and subsequently for 10 minutes at 55°C in 1 × SSC, 1% SDS. The membranes were then exposed to autoradiography on an X-ray film for 30 minutes, 3 hours and 70 hours.

## Results

To test whether the PCR protocol described here detects all *C. trachomatis* serovars relevant for urogenital infections, serovars D-L2 grown on McCoy cells were subjected to PCR. 0.2 ml of cell culture supernatant was centrifuged at 3000 *g* for one hour at room temperature and the resulting pellet resuspended in 7 µl culture medium. After boiling for 15 minutes PCR was performed. Amplification products were electrophoretically separated in a 2% agarose gel stained in ethidium bromide and blotted on Gene Screen plus membranes. These were

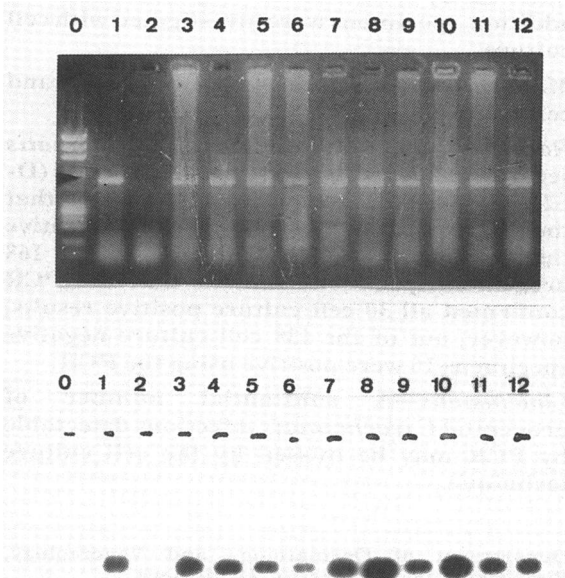


Fig 1 Detection of serovar D-L2 by PCR. 0 =  $\phi$ X-marker; 1 = positive control (suspension of serovar L2); 2 = negative control (McCoy cells); 3-12 = serovars D-L2. Ethidium bromide stained agarose gel (top); autoradiogram after Southern blot hybridization with the diagnostic oligonucleotides (bottom).

hybridised with the diagnostic oligonucleotide. All serovars could be specifically amplified (fig 1).

Next the sensitivity of the PCR-protocol was tested in comparison with the routinely used cell culture system for the detection of *C trachomatis*. A culture suspension containing living organisms of *C trachomatis* serovar L2 was serially diluted and aliquots of each dilution step were investigated in parallel with the cell culture and PCR technique. DNA of the obligatory *C trachomatis* plasmid pCTT1 could be demonstrated even with a dilution of  $10^{-5}$  whereas inclusions could only be detected when cell culture was inoculated with a dilution of at least  $10^{-3}$ .

Investigation of 30 culture-positive urogenital specimens revealed that all were positive with the PCR. When the PCR was performed for 138 culture-negative specimens, 16 of them (11.6%) were positive. As demonstrated for one of the series of investigation distinct bands of the amplified 528 base pair fragment could be visualised in ethidium bromide stained agarose gels (fig 2). Subsequent hybridisation of Southern blots confirmed the amplified respective *C trachomatis* plasmid pCTT1 sequences.

## Discussion

The evaluation of methods to directly detect *C trachomatis* by fluoresceine-labelled monoclonal antibodies, enzyme immunoassay or nucleic acid hybridisation indicated that at least one portion of the results which are discrepantly positive with respect to cell culture mean actual infections.<sup>13</sup> Accordingly,

even before the introduction of tests to detect chlamydiae directly, it was generally thought that the sensitivity of cell cultures reaches only 90%.<sup>17-19</sup> Owing to a limited sensitivity of the tests to directly detect chlamydiae this assumption could not be proved formally. Our study and a previous similar one<sup>20</sup> make clear that, compared to cell culture, amplification of defined sequences of *C trachomatis* and the consecutive demonstration of these sequences by electrophoresis and hybridisation lead to a higher sensitivity of detection of *C trachomatis* in urogenital specimens.

Moreover, a simplified protocol allowed the detection of *C trachomatis*-DNA without prior extraction of nucleic acids: Boiling of the specimens proved sufficient to obtain results comparable with those obtained with purified DNA.<sup>20</sup> On the one hand, fewer procedure steps reduce the risk of contamination, representing the most important problem of the PCR-technique. On the other hand, the simplified protocol is faster, thus allowing the investigation of larger numbers of specimens.

The detection of *C trachomatis* by our PCR protocol is based on the amplification of the DNA-sequence of the third open reading frame of the *C trachomatis* plasmid pCTT1. Whereas this plasmid could not be detected in the species *C psittaci* and *C pneumoniae* as well as in the mouse pneumonitis biovar of *C trachomatis*, it was found to be present in all strains of the trachoma and LGV biovars investigated so far.<sup>22-24</sup> Accordingly, this PCR protocol allowed the amplification and detection of the defined plasmid DNA-sequence in suspensions of cultures inoculated with serovars D-L2. Similarly using a PCR-technique based on amplification of DNA-sequences located at almost the same region in the third open reading frame of pCTT1, Ostergaard *et al*<sup>20</sup> detected amplified plasmid-DNA in a panel of *C trachomatis* prototype serovars. Moreover, Palmer and Falkow<sup>23</sup> showed that the DNA-sequences of this reading frame is regularly transcribed in mRNA during the intracellular replication of *C trachomatis*. Therefore expression of pCTT1 genes might be an essential prerequisite for the replication of the host organism. Further studies using reverse PCR protocols are presently performed to investigate the pathogenetic role of the pCTT1 plasmid in *C trachomatis* infections.

Serial dilution experiments revealed that the detection of *C trachomatis* using this PCR protocol was 100 fold increased compared to the conventional cell culture technique. The urogenital specimens taken to evaluate the PCR had been investigated with cell culture carried out in vials, and for the demonstration of inclusions fluoresceine conjugated monoclonal antibodies had been used, that is, cell culture had been performed to reach the optimal sensitivity of this method. Nevertheless in 16 of 138 culture

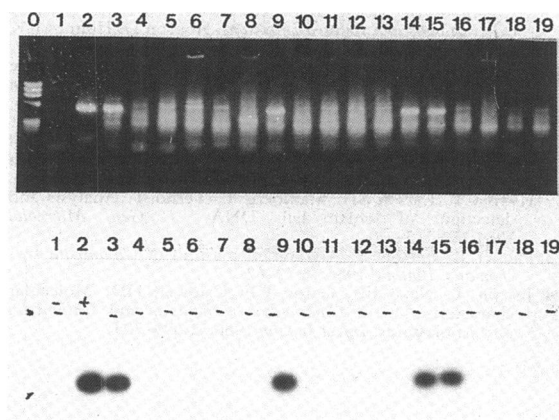


Fig 2 Investigation of a series of culture negative urogenital specimens by PCR. 0 =  $\Phi$ X-marker; 1 = negative control (Mycobacterium cells); 2 = positive control (suspension of serovar L2); 3-19 = culture negative urogenital specimens. Specimens positive by PCR: 3, 9, 14, 15. Ethidium bromide stained agarose gel (top); Autoradiogram after Southern blot hybridisation with the diagnostic oligonucleotides (bottom).

negative specimens chlamydial plasmid sequences could be demonstrated by the PCR, indicating that a substantial number of urogenital *C trachomatis* infections may be missed by the cell culture technique, presently the method of choice.

The clinical relevance of the detection of chlamydial DNA by PCR in urogenital specimens which were negative with cell culture remains to be defined. On the one hand, *C trachomatis* plasmid-DNA could be a residue of a recent however already healed infection. In this case the detection of chlamydial DNA with the PCR would be without any therapeutic relevance. On the other hand, the PCR might detect infections with replicating *C trachomatis* organisms beyond the sensitivity of the cell culture technique. Finally, failure of the cell culture might indicate a latent phase of the infection without replication of *C trachomatis*. In this case PCR might be the only diagnostic procedure to reveal the infection and may be the appropriate method for the follow up of therapy. Antibiotic treatment initiated because of the positive result of the PCR alone, however, may remain ineffective in these cases, since the therapeutic success of the antibiotics depends on the replication of *C trachomatis*. Additional studies will have to be undertaken to clarify the clinical relevance of *C trachomatis* detection by PCR.

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- 1 Ridgway GL, Oriel JD, Mumtaz G, Mellars B. Comparison of methods for detecting *Chlamydia trachomatis*. *J Clin Pathol* 1985;39:232-3.
- 2 Stephens RS, Kuo CC, Tam MR. Sensitivity of immunofluorescence with monoclonal antibodies for detection of *Chlamydia trachomatis* inclusions in cell culture. *J Clin Microbiol* 1982;16:4-7.
- 3 Horn JE, Quinn T, Hammer M. Use of nucleic acid probes for the detection of sexually transmitted infectious agents. *Diagn Microbiol Infect Dis* 1986;4:101-9.
- 4 Näher H, Petzoldt D, Sethi KS. Evaluation of non-radioactive in situ hybridisation method to detect *Chlamydia trachomatis* in cell culture. *Genitourin Med* 1988;64:162-4.
- 5 Tam MR, Stamm WE, Handsfield HH, et al. Culture-independent diagnosis of *Chlamydia trachomatis* using monoclonal antibodies. *N Engl J Med* 1984;310:1146-50.
- 6 Nowinski RC, Tam MR, Goldstein LC, et al. Monoclonal antibodies for diagnosis of infectious diseases in humans. *Science* 1983;219:637-44.
- 7 Näher H, Petzoldt D. Evaluation of an enzyme immunoassay (Chlamydiazyme) and a direct immunofluorescence technique (MicroTrak) for the detection of *Chlamydia trachomatis* antigen in urogenital specimens. *Euro J Sex Transm Dis* 1986;3:217-22.
- 8 Caldwell HD, Schachter J. Immunoassay for detecting *Chlamydia trachomatis* major outer membrane protein. *J Clin Microbiol* 1983;18:539-45.
- 9 Jones MF, Smith TF, Houghlum AJ, Herrmann JE. Detection of *Chlamydia trachomatis* in genital specimens by the chlamydiazyme test. *J Clin Microbiol* 1984;20:465-7.
- 10 Näher H, Niebauer B, Hartmann M, Söltz-Szöts J, Petzoldt D. Evaluation of a radioactive rRNA:cDNA-hybridisation assay for the direct detection of *Chlamydia trachomatis* in urogenital specimens. *Genitourin Med* 1989;65:319-22.
- 11 Näher H, Niebauer B, Hartmann M, Söltz-Szöts J, Petzoldt D. Nukleinsäure-Hybridisierung zum Direktnachweis von *Chlamydia trachomatis*. *Hautarzt* 1990;41:151-4.
- 12 Pao CC, Lin SS, Yang TE. Deoxyribonucleic acid hybridization analysis for the detection of urogenital *Chlamydia trachomatis* infections in women. *Am J Obstet Gynecol* 1987;156:195-9.
- 13 Ridgway G. The laboratory diagnosis of chlamydial infection. Oriel D, Ridgway GL, Schachter J, Taylor-Robinson D, Ward M eds. *Chlamydial infections*. Cambridge University Press 1986;S539-549.
- 14 Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* 1987;155:335-50.
- 15 Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988;239:487-91.
- 16 Maniatis T, Fritsch EF, Sambrook J. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY. Cold Spring Harbor Laboratory, 1982.
- 17 Schachter J. Immunodiagnosis of sexually transmitted disease. *Yale J Biol Med* 1985;58:443-7.
- 18 Ripa TH. Microbiological diagnosis of *Chlamydia trachomatis* infection. *Infection* 10 (Suppl. 1) 1982:19-23.
- 19 Wentworth WB. Sensitivity of cell culture for isolation of *Chlamydia trachomatis* from genital sources. In: *Non-gonococcal Urethritis and Related Infections*. Hobson D, Holmes KK eds, Washington DC. *Am Soc Microbiol* 1977;S:299-303.
- 20 Ostergaard L, Birkelund S, Christiansen G. Use of polymerase chain reaction for detection of *Chlamydia trachomatis*. *J Clin Microbiol* 1990;28:1254-60.
- 21 Dutilh B, Bébér C, Rodriguez P, Vekris A, Bonnet J, Garret M. Specific amplification of a DNA sequence common to all *Chlamydia trachomatis* serovars using the polymerase chain reaction. *Res Microbiol* 1989;140:7-16.
- 22 Hyypä T, Larsen SH, Ståhlberg T, Terho T. Analysis and detection of chlamydial DNA. *J Gen Microbiol* 1984;130:3159-64.
- 23 Palmer L, Falkow S. A common plasmid of *Chlamydia trachomatis*. *Plasmid* 1986;16:52-62.
- 24 Joseph T, Navo FE, Garon CF, Caldwell HD. Molecular characterisation of *Chlamydia trachomatis* and *Chlamydia psittaci* plasmids. *Infect Immun* 1986;51:699-703.